PATENT

Docket No. 1377-0137P

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS:	John Kevin Collins *et al.*	
APPLN. NO.:	09/367,105	GROUP: 1651
FILED:	November 10, 1999	EXAMINER: I. Marx
FOR:	PROBIOTIC STRAINS FROM LACTOBACILLUS SALIVARIUS AND ANTIMICROBIAL AGENTS OBTAINED THEREFROM	

DECLARATION UNDER 37 C.F.R. § 1.32

Assistant Commissioner of Patents

Washington, DC 20231

Sir:

I, Liam O'Mahony, Ph.D., am presently employed at the Virology/Immunology Unit, Department of Microbiology, University College Cork - National University of Ireland, Cork, College Road, Cork, Ireland. My *Curriculum Vitae* is attached hereto. I do solemnly and sincerely declare as follows:

1. I am authorized to make this Declaration on behalf of the Applicants.

2. I have read the specification of the above U.S. Application and I have been provided with a copy of the Office Action dated June 19, 2001 and the cited references relied on by the Examiner. I am also fully familiar with the invention the subject of the Application. I have noted the views expressed by the Examiner on each of the cited references. When assessing the invention the subject of the present Application in the light of the prior art relied on it is important to be aware that there can be significant differences in biological properties between strains.

3. The advantages of using the particular strains of the present invention include:

   1) species-specificity;

   2) the strains are selected/adapted to the human environment; one would not expect adaptation in any other environment; and

   3) they are host specific.

4. It is submitted that strains having those advantages are neither disclosed or suggested in any of the documents cited by the Examiner.

5. Another important aspect of the invention is the source of the *Lactobacilli.* These *Lactobacilli* are adherent to human gastrointestinal tissue from subjects with healthy gastrointestinal tracts and no associated pathology. Isolation of *Lactobacilli* from faeces is entirely different. The fecal flora represents the luminal contents of the distal large bowel whereas the mucosa adhering microflora represents a highly specialised microenvironment. Adherent strains must be able to survive a more aerobic environment than that present in the lumen. In addition, adherent strains must survive and thrive in an immunologically hostile environment. The inventors aimed to and succeeded in isolating *Lactobacilli* with immunomodulatory properties which has not previously been reported in the prior art. No strains have previously been isolated in this manner followed by *in vitro* and *in vivo* characterisation.

6. None of the cited documents discloses or suggests a strain of *L. salivarius* having the properties set forth in claim I. In particular none of those documents discloses or suggests a strain of *L. salivarius* isolated from resected and washed human gastrointestinal tract and which is thus adherent to human gastrointestinal tract.

7. As emphasised above the strains of *Lactobacillus salivarius* claimed in claim 1 are isolated from "resected and washed human gastrointestinal tract". The strains are intended for use as probiotic agents in humans. Thus, the strains must meet certain

criteria if they are to be capable of being used as probiotics; more particularly they must meet the criteria laid down by the Lactic Acid Bacteria Industrial Platform (LABIP; Guarner and Schaafsma, "Probiotics" *Int. J. Food Microbiol* 1998; 39:237-238; see Annex I) and others for the selection of probiotic microorganisms intended for use in humans. Reference is also made to Tannock (1997; TIBTECH 15: 270-274 "Probiotic properties of lactic acid bacteria: plenty of scope for fundamental R & D"), particularly at page 272 second column (see Annex II).

8. Arihara deals with a strain of *L. salivarius* which has been identified biochemically (using its fermentation profile) as a strain of *Lactobacillus salivarius* subsp. *salicinius*. *Lactobacillus salivarius* subsp. *salicinius* strain T140 was isolated from the "surface of Japanese pampas grass" which may have been contaminated by feces excreted by a domesticated animal. The Examiner states that these strains were isolated from the human gastro-intestinal tract. However, this is incorrect as no attempt was made to isolate lactic acid bacteria from washed and resected gastrointestinal tissue.

9. In contrast, the strains of *L salivarius* claimed in the present Application were deliberately isolated from the human GI tract (i.e. the environment in which they will be required to function) in order to ensure compliance with the recommended criteria laid down by the aforementioned LABIP. This is not the case

with the strain of *Lactobacillus salivarius* subsp. *salicinius* disclosed in Arihara *et al.*, nor is there any suggestion therein of such isolation.

10. Arihara *et al* describe 353 bacterial strains isolated from food, plants, saliva or animal feces. The Examiner takes the view that the cited reference discloses a *Lactobacillus salivarius* which appears to be identical to the presently claimed strain and refers for example to Table 1 on page 421. This is not the case for the reasons stated above. Furthermore, a number of the reported strain characteristics are different to those described by Collins *et al.* The assumptions made by the Examiner that the microorganisms disclosed in Arihara may be correct in that these bacterial strains may survive passage through the human gastrointestinal tract. However, it is incorrect to assume that these bacterial strains could exert any influence on the gastrointestinal microflora, or that these bacterial strains could interact with the human host resulting in certain health benefits. The fecal flora represents the luminal contents of the distal large bowel whereas the mucosa adhering microflora represent a highly specialised microenvironment. Adherent strains must be able to survive a more aerobic environment than that present in the lumen. In addition, adherent strains must survive and thrive in an immunologically hostile environment. These adherent bacteria must interact with the host immune system in order to survive and therefore will be immunomodulatory in nature.

11. The primary aim of the isolation process described in the present Application invention was to isolate bacteria which come into direct contact with human epithelial cells, with potent anti-microbial activity but which do not elicit a pro-inflammatory immunological response. Bacteria present within luminal contents do not necessarily interact with the gastrointestinal mucosa.

12. The inventors took the view that truly probiotic bacteria should be isolated only when adherence to the gastrointestinal tract can be demonstrated. However, Arihara *et al.* refers to probiotic bacteria isolated from other environments. In addition, Arihara *et al.* do not refer to the relevant factors affecting bacterial survival in the human gastrointestinal tract, such as the mucosal immune system. The state of the art prior to the invention did not include the concept that complex intimate molecular interactions between the host and bacterium would be required to induce probiotic health benefits.

13. The Examiner states that Arihara *et al.* also disclose a product having broad spectrum antimicrobial activity. The bacteriocin produced by the strain disclosed in Arihara *et al.* inhibits the growth of other closely related lactobacilli (Table 2, page 422) whereas strains described in the present Application importantly do not.

14 The strains of *L. salivarius* claimed in the present Application have the ability to selectively kill pathogenic bacteria without killing off many closely related lactobacilli which have health--promoting properties and with which they exist in symbiotic relationship. *Lactobacillus salivarius* subsp. *salicinius* T140 differs from the presently claimed strains in not meeting the recommended criteria for the selection of probiotic strains proposed *inter alia* by LABIP.

15. This is an important trait as one would require a probiotic bacterium to antagonise the growth of pathogenic species but not affect the composition of the commensal flora. The isolation of *Lactobacillus salivarius* species from resected and washed human tissue resulted in the identification of strains with this trait. Environment pressures resulting in the selection of strains most suited to survive and thrive in that environment are obviously completely different between grass and the human gastrointestinal tract. Furthermore, salivacin 140, produced by the Arihara *et al.* strain, requires a high initial pH for production while the antimicrobial factors produced by the strains described in the present Application do not require such a high initial pH. These strain dependent differences demonstrate that the strains described in the present Application are novel and have not been previously described. In fact, Arihara *et al.* state "Thus salivacin 140 production is a strain-specific phenomena like most cases of the bacteriocin synthesis by lactic acid bacteria." (*sic*).

16. Ten Brink *et al.* disclose approximately 1000 lactobacillus strains isolated from fermented foods and feeds, human dental plaque and feces from laboratory animals and humans. The rationale underlying the isolation and screening programme described by ten Brink *et al.* is directed to the identification of lactic acid bacteria with anti-microbial properties suitable for use in food preservation. Thus, it was not suggested or anticipated by these authors that these isolates could be active within the human gastrointestinal tract by influencing pathogen adhesion or invasion. The strains of *L. salivarius* claimed in the present Application have been identified by means of biochemical and SDS-P AGE analysis as strains of *Lactobacillus salivarius* subsp. *Salivarius*. Thus, the respective strains are different. In fact, it is highly unlikely that these isolates would provide any health benefits to humans.

17. Two *Lactobacillus* strains are described in further detail in the ten Brink *et al.* reference. A *Lactobacillus salivarius* strain and a *Lactobacillus acidophilus* strain were reported to produce anti-microbial compounds designated salivaricin B and acidocin B, respectively. The *Lactobacillus acidophilus* strain is not considered further as it is a different species to those described by the present Application. *Lactobacillus salivarius* M7 produced salivaricin B which was active primarily against related lactobacilli. This is in contrast to the results described in the Application. In addition, salivaricin B is not heat stabile

while ABP118 is heat stable. Unlike the antimicrobial agent ABP 118 of the present Application, acidocin B produced by *Lactobacillus acidophilus* M46 of ten Brink *et al.* fails to retain any activity following the heat treatment at 121 °C. Under such conditions the antimicrobial agent ABP 118 retains at least 50% of its activity (see Table 9 of the specification of the present Application). The strains of *L. salivarius* claimed in respect of the present Application are identified *inter alia* by the fact that the secretory products produced thereby are maintained in the presence of physiological concentrations of human bile and human gastric juice. Thus, the strains are resistant to both bile and gastric acid, one of the recommended criteria of LABIP. Each of strains *Lactobacillus acidophilus* M46 and *Lactobacillus salivarius* M7 described in ten Brink was isolated from human dental plaque, not resected and washed human tissue, or grass near a barn as stated by the Examiner. The Examiner also states that these strains are likely to live within the human gastrointestinal tract. However, no studies were performed to assess the acid and bile tolerances of these strains. In fact, survival within the environment of dental plaque would suggest that these strains would not survive lower gastrointestinal tract transit. In addition, the bacteria associated with human dental plaque induce inflammatory responses. Adherent strains from resected and washed tissue would not be expected to induce inflammatory responses. Thus, the strains claimed in the present Application, and their anti-microbial factors, have not been previously described.

18. Suhr-Jessen *et al.* describe the isolation of lactic acid bacteria from the pig gastrointestinal tract. The Examiner draws particular attention to examples 4 and 5 which describe the acid and bile tolerances of the strains isolated from the pig gastrointestinal tract. These assays were not carried out using human bile or human gastric juice. The composition of human bile, and thereby the antagonistic activity of human bile, is distinct from Bactooxgall used by Suhr-Jessen *et al.* Collins *et al.* describe lactobacillus strains isolated from resected and washed human gastrointestinal tract that survive, and maintain their anti-microbial activity, in human bile and gastric juice. The panel of lactic acid bacteria, described by Suhr-Jessen *et al.*, were selected to be included in fermented milk products intended for human consumption or in veterinary compositions for treating gastrointestinal diseases. However, the inventors did not consider the intimate interactions that occur between a newly ingested bacterium and its specific host environment. These interactions depend on a number of factors including the microflora already present, species-specific attachment sites (e.g. complementary bacterial-epithelial cell structures), species-specific immunological parameters, the health status of the host, current medications, etc. There are similarities between the human and porcine digestive systems. Indeed, the ability of a bacterium to survive passage through the pig intestine renders probable the ability to survive passage through the human gut as well. However, one cannot predict the intimate interactions

between a newly ingested bacterium and its specific host environment. These interactions depend on the various factors enumerated above. In addition, the literature illustrates many examples of bacterial and viral pathogens that are host specific – at least with regard to the severity of infection. Thus, by the same logic, it can be stated that bacterial-host interactions can be species specific and for human purposes bacterial strains must be isolated from the human gastrointestinal tract. It is not sufficient to extrapolate from animal studies to the human situation. It would not have been obvious in my opinion to Suhr-Jessen *et al.* that species-specific interactions determine the probiotic potential of a newly isolated strain.

19. I have carried out a series of experiments under *in vitro* and *in vivo* conditions which demonstrate the feature of adhesion of a strain according to the invention, the subject of the above-identified application. Results of these experiments can be summarized as follows.

20. <u>*In vitro* adhesion assays</u>

Lactobacillus salivarius strain UCC118 (NCIMB 40829) was co-incubated with human gastrointestinal epithelial cell lines (Caco-2 and HT-29). Following a short incubation period, these cell lines were washed vigorously and examined for the presence of adherent bacteria. Significant adherence of *Lactobacillus salivarius* strain UCC118 (NCIMB 40829) to

these human epithelial cell lines was noted. Following adherence of NCIMB 40829 to epithelial cells, invasion by enteropathogenic *Listeria monocytogenes*, *Salmonella typhimurium* and *Shigella flexneri* into epithelial cells was significantly reduced. However, NCIMB 40829 itself did not invade the epithelial cells. Analysis of epithelial cell gene expression following bacterial adhesion revealed profound alterations in the expression of genes regulating mucosal integrity. A partial amino acid sequence of the bacterial adhesin has been obtained, while one of the eucaryotic receptors for NCIMB 40829 has been identified. Following consumption of this strain in a murine model of colitis, significant alterations in the gut flora were associated with reduced gastrointestinal inflammatory activity and tumour incidence (O'Mahony *et al.*, Alimentary Pharmacology and Therapeutics, 2001).

21. *In vivo* adhesion assays

Lactobacillus salivarius strain UCC118 (NCIMB 40829) was consumed by healthy human subjects in a placebo controlled randomised study. This bacterium was delivered in high numbers to the gastrointestinal tracts of healthy study subjects by both fresh milk and fermented milk (yoghurt) products. Prior to the feeding period, no bacteria were isolated from subject feces on MRS medium containing rifampicin. After feeding, 39 of the 40 volunteers fed with *Lactobacillus salivarius* strain UCC118 (NCIMB 40829)-containing product (10^{10} CFU/day, for 21 days) exhibited significant faecal excretion (10^3 to 10^7

cfu/g wet weight) of rifampicin resistant lactobacilli. These bacteria were confirmed to be *Lactobacillus salivarius* strain UCC118 (NCIMB 40829) on the basis of production of the ABP118 peptide, antibiotic resistance, and antibacterial profile. As expected, excretion of *Lactobacillus salivarius* strain UCC118 (NCIMB 40829) was not detected in the control-fed subjects (n=40). Of the volunteers fed test product (n=40), five individuals (12.5%) were found to have significant fecal concentrations of the *Lactobacillus salivarius* strain UCC118 (NCIMB 40829) 21 days after termination of feeding. 100 days after the feeding period finished, one individual in the fermented milk delivery group was still excreting detectable fecal levels of this bacterium. As gastrointestinal transit time averages 1 to 3 days, this is definitive proof of *Lactobacillus salivarius* strain UCC118 (NCIMB 40829) adhesion to and survival within the human gastrointestinal tract associated with mucosal immunological tolerance of the consumed strain.

22. A pilot, non-placebo controlled trial on feeding NCIMB 40829 to patients with Crohn's disease has been completed. Crohn's disease is an inflammatory disorder of the gastrointestinal tract with unknown aetiology. Following consumption of this bacterial strain for 6 weeks, patients immunologically perceived the presence of NCIMB 40829 due to the appearance of antibodies specific to this strain. However, this was not a pro-inflammatory response. In fact, a significant proportion of

patients avoided steroid therapy and patient disease scores (CDAIs) significantly decreased.

23. The isolation of adherent micro-organisms from washed and resected gastrointestinal tissue according to the invention has resulted in the isolation of non-pathogenic bacteria with significant health benefits in humans. All the effects of NCIMB 40829 mentioned above are a function of the capability of this micro-organism to adhere to gastrointestinal epithelium *in vivo*.

I hereby declare that all statements made herein of my own knowledge are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

5th Dec 2001

Date

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Douglas Community School 1985-1990

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Publications.

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Referees.

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APPENDIX I

ELSEVIER

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International Journal of Food Microbiology

Short communication

Probiotics

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for LABIP Workshop participants[1]. [a]Digestive System Research Unit, Hospital General Vall d'Hebron, 08035 Barcelona, Spain
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There is currently a growing interest in certain lactic acid bacteria strains that have been suggested or shown to provide specific health benefits when consumed as food supplements or as food components. However, opinions differ widely with respect to the requirements needed to substantiate a claim on a beneficial effect of a given bacterial strain, and there is no consensus on how to define and accredit a viable strain as a probiotic. On the other hand, putative risks of massive introduction of new live microrganisms in nutrition should also be envisaged, even if benefits were proven. The Lactic Acid Bacteria Industrial Platform (LABIP)[2] hosted a workshop sponsored by the European Community to discuss these topics.[3]

Firstly, the workshop issued a consensus definition of probiotics: "oral probiotics are living micro-organisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition". According to the new definition, probiotics may be consumed either as a food component or as a non-food preparation.

For the demonstration of probiotic activity of a certain strain, the group concluded that well-designed human studies (double blinded, placebo-controlled) are required. Several in vitro assays or animal studies such as tests on resistance to bile and acid, adhesion to the intestinal mucosa, effects on immunocompetent cells or antimutagenicity, are very useful in the preselection of bacterial strains. However, the proof of efficacy in humans should be granted by at least one well-designed human study. Preferentially, the study should be published in a peer-reviewed journal.

Based on these criteria, the group discussed and proposed to make a distinction between established beneficial effects of probiotics, and potential benefits that need further substantiation. For instance, several lines of evidence have established the benefits of

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[1]List of LABIP Workshop participants: Dr. W. Boersma (Leiden, Netherlands), Prof. J.K. Collins (Cork, Ireland), Dr. M. Coste (Jouy-en-Josas, France), Dr. I. de Smet (Gent, Belgium), Dr. F. Guarner (Barcelona, Spain), Prof. W. Hammes (Stuttgart, Germany), Dr. T. Matilla-Sandholm (Helsinki, Finland), Prof. L. Morelli (Piacenza, Italy), Dr. B.L. Pool-Zobel (Karlsruhe, Germany), Dr. I.R. Rowland (London, UK), Prof. G.J. Schaafsma (Zeist, Netherlands), Prof. K.H. Schleifer (München, Germany), Dr. M. Tvede (Vibourg, Denmark), Prof. T. Wadstrom (Lund, Sweden), Dr. B. Weile (Gentofte, Denmark). Chairman: Dr. J.W. vd Kamp (Zeist, Netherlands). EU-representative: Dr. A. Aguilar (Brussels, Belgium).

[2]LABIP is an European Economical Association of companies involved in the use and production of lactic acid bacteria (LAB). Its goals are to promote R and D on LAB within the EU and to develop an opinion on research needs for LAB within the EU.

[3]The Workshop on Probiotics was held in Frankfurt (Germany) from 13 to 15 November 1995.

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certain probiotics to reduce signs and symptoms of lactose intolerance (Sanders, 1993), prevention and treatment of certain diarrhoeal diseases (Biller et al., 1995; Kaila et al., 1995; Majamaa et al., 1995; Saavedra et al., 1994; Siitonen et al., 1990), reduction of bacterial enzyme activities (Sanders, 1993) and stimulation of the immune system (De Simone et al., 1993; Schiffrin et al., 1995). Potential benefits of the ingestion of probiotics can also be expected in other important fields such as modulation of blood cholesterol levels, competitive exclusion of intestinal pathogens, and cancer prevention.

The unlimited use of probiotics might have unwanted side-effects. Most likely, these effects would not affect the normal healthy population, but should be considered when used by specific subgroups of persons 'at risk'. For instance, infection and toxicity by probiotics has never been documented, but subjects with underlying disease conditions that predispose to infection might be exposed to a putative risk (Adams and Marteau, 1995). Likewise, unrestricted stimulation of the immune system by probiotics could be detrimental for patients suffering autoimmune diseases. The risk of transfer of antibiotic resistance properties from probiotics to virulent micro-organisms should also be evaluated.

Studies on probiotics that will reasonably expand our knowledge in this emerging field should be encouraged for active research in forthcoming years.

A copy of the full report of the Workshop is available from the participants.

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APPENDIX II

Probiotic properties of lactic-acid bacteria: plenty of scope for fundamental R & D

Gerald W. Tannock

Probiotic products are marketed widely throughout the world. This is especially true of yogurts that contain strains of lactic-acid bacteria of intestinal origin. Consumption of these products is aimed at promoting the wellbeing of the consumer by impacting on the collection of microorganisms that normally inhabit the intestinal tract. The development of scientifically valid probiotics requires more detailed knowledge of this intestinal microflora than is currently available.

The term 'probiotic' originally referred to a phenomenon observed when two organisms were cultured together, in which substances produced by one organism stimulated the growth of the other organism. These growth-promoting substances were referred to as 'probiotics'[1]. The term was subsequently used to describe living preparations of microbial cells that could be administered to animals, including humans, with the aim of promoting the health of the consumer[2]. This latter concept is derived from the rather philosophical observations made by Elie Metchnikoff and others earlier this century (Box 1). In the case of farm animals, faster weight gain for the same amount of food consumed (growth promotion, feed efficiency) have been of primary importance.

By definition, a probiotic is a 'live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance'[2]. The marketing of probiotics for human consumption relies heavily on this definition. Specific health-related claims are not usually made in relation to the products, but general statements such as 'helps maintain a healthy balance of beneficial bacteria', 'stabilizes the intestinal microflora and modulates its function', 'promote the positive balance of the intestinal flora' are included on labels and in advertising brochures. Implicit in the definition and in the marketing of probiotics is that the ingested microorganisms impact on the composition of the normal microflora of the intestinal tract. The manufacture and marketing of probiotic yogurts (Acidophilus–Bifidus yogurts) has increased dramatically worldwide in recent years, and a desire to ascribe some scientific validity to these products, rather than to rely on a theoretical concept, is apparent in the dairy-food industry. Establishing the scientific validity of probiotics will require a substantial commitment from industry to supporting fundamental studies of microorganism–host relationships. The results of these fundamental studies will define the health-related aspects in relation to lactic-acid bacteria that should be studied in the future.

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The intestinal microflora

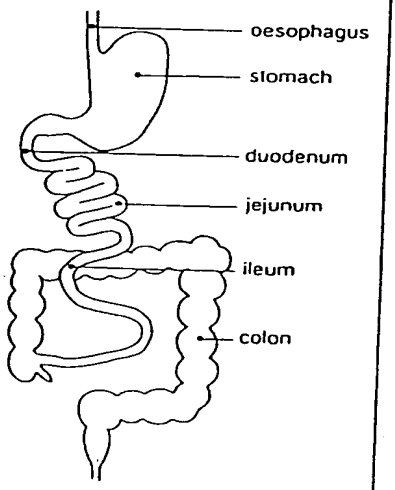
The intestinal tracts of all mammalian species that have been studied, appropriately harbour a complex collection of microorganisms, mostly anaerobic bacterial species, that is known as the intestinal microflora[3]. This microflora is mostly located in the large bowel in monogastric animals such as humans (Box 2). The predominant bacterial members of the microflora attain population levels of about 10^{10} per gram (wet weight) of contents, so that the human colon, for example, contains at least 10^{12} living bacterial cells. About 400 bacterial species have been detected in human faeces (the faecal microflora is representative of the microflora of the colon[6]) but 30 to 40 species constitute 99% of the collection in any one human subject[7]. While the numerically predominant genera of bacteria detected in the faeces of different individuals are the same, there is variation in the occurrence and population size of bacterial species[6]. The complex composition of the intestinal microflora is even more apparent from the results of recent studies in which populations of bacteria were analysed at the level of bacterial strains. Species can be divided into numerous strains on the basis of differences in the occurrence of short sequences of genomic DNA at which enzymes (restriction endonucleases) will cut the polynucleotide strands. Variation in the number and location of these sequences in different strains provides

Box 1. Elie Metchnikoff (1845–1916) and the elixir of life

Elie Metchnikoff, Nobel Laureate (for his discovery of phagocytosis) of the Institut Pasteur, Paris, was interested in the scientific basis of ageing. Couched in the natural history perception of science prevalent in his time, Metchnikoff's books convey an overall message that the intestinal microflora could be detrimental to the host. According to Metchnikoff, the large bowel harboured microorganisms that produced substances that were toxic to the vascular and nervous systems. The toxic substances, as a result of absorption into the bloodstream, contributed to the ageing process. Thus, intestinal microorganisms were the aetiological agents of 'autointoxication' because they produced ammonia, amines and indole as a result of protein hydrolysis (putrefaction) in the digestive tract. Metchnikoff's remedy for autointoxication was radical: he advocated surgical removal of the large bowel. However, a more acceptable remedy was to modify the intestinal microflora by replacing or diminishing the number of putrefactive microorganisms in the intestine. This could be accomplished, it was suggested, by enriching the microflora with bacterial populations that obtained energy by the fermentation of carbohydrates rather than hydrolysis of proteins. Lactic-acid-producing bacteria were favoured as fermentative microorganisms for this purpose because it had been observed that the natural fermentation of milk by these microorganisms prevented the growth of non-acid-tolerant microorganisms, including those with proteolytic activity. If a lactic fermentation prevented the putrefaction of milk, would it not have the same effect in the digestive tract if appropriate microorganisms were used? The inhabitants of Eastern European countries, some of whom were, apparently, extremely long-lived, consumed fermented milk as a constant part of their diet. Thus, yogurts were introduced to Western Europe as health-related foods[3,4].

Box 2. Bacterial genera that are commonly detected as components of the intestinal microflora of humans

Bacteroides
Gram-negative, non-spore-forming bacilli. Obligate anaerobes. Metabolic products include combinations of acetic, succinic, lactic, formic or propionic acids. If *N*-butyric acid is produced, isobutyric and isovaleric acids are also present.



Bifidobacterium
Gram-positive, non-spore-forming, nonmotile bacilli, sometimes with club-shaped or spatulated extremities. Obligate anaerobes. Acetic and lactic acids are produced primarily, in the molar ratio 3:2. Glucose is degraded exclusively and characteristically by the fructose-6-phosphate 'shunt' metabolic pathway.

Clostridium
Gram-positive bacilli that form endospores. Obligate anaerobes.

Enterococcus
Gram-positive cocci. Facultative anaerobes. Lancefield group D. Can grow in 6.5% NaCl broth and in normal broth at pH 9.6.

Eubacterium
Gram-positive bacilli, non-spore-forming. Obligate anaerobes. Produce mixtures of organic acids including butyric, acetic and formic acids.

Fusobacterium
Gram-negative, non-spore-forming bacilli. Obligate anaerobes. *N*-butyric acid is produced but isobutyric and isovaleric acids are not.

Peptostreptococcus
Gram-positive cocci. Obligate anaerobes. Can metabolize peptone and amino acids.

Ruminococcus
Gram-positive cocci. Obligate anaerobes. Amino acids and peptides are not fermented. Fermentation of carbohydrates produces acetic, succinic and lactic acids, ethanol, carbon dioxide and hydrogen.

Lactobacillus
Gram-positive bacilli, non-spore-forming. Grow best under anaerobic conditions. Lactic acid is a major product of glucose fermentation.

Escherichia coli
Gram-negative rods, facultatively anaerobic. Citrate not utilized. Carbohydrates fermented to lactic, acetic and formic acids. Part of formic acid is split by a complex hydrogenase system to give equal amounts of carbon dioxide and hydrogen. Lactose is fermented by most strains but fermentation can be delayed or absent. Motile by means of peritrichous flagella or nonmotile.

Box 3. You can be identified on the basis of your intestinal microflora

In recent studies carried out in the author's laboratory, the compositions of the bifidobacterial and lactobacillar populations inhabiting the intestine of ten healthy humans were analysed by differentiating between bacterial strains according to their genetic fingerprints. Faecal homogenates were prepared, diluted serially and selective agar plate cultures set up. After incubation and enumeration of the cultured bacteria, randomly selected colonies were subcultured and fingerprinted. The proportion that each strain contributed to the total bifidobacterial or lactobacillar population was thus determined. Faecal samples were collected from two of the subjects at monthly intervals over a 12-month period. These studies revealed that each human had their own unique collection of *Bifidobacterium* and *Lactobacillus* strains. In some subjects, a relatively simple collection of strains was present; in others it was complex. Subject 1, for example, harboured a simple and constant collection of bifidobacterial strains (Fig. 1) whereas Subject 2 had a complex bifidobacterial microflora that appeared to fluctuate in composition (Fig. 2)[8]. (Figures reproduced courtesy of the American Society for Microbiology.)

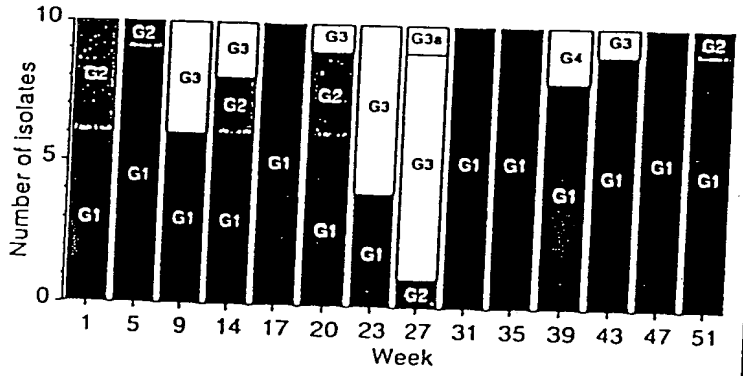


Figure 1

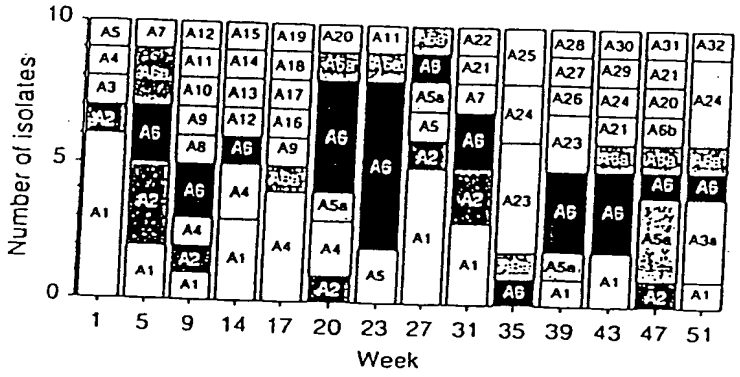


Figure 2

a method of genetic fingerprinting: DNA from a particular bacterial isolate, when digested with an appropriate restriction endonuclease, gives a characteristic pattern of different-sized DNA fragments after agarose-gel electrophoresis. Molecular analysis of two lactic-acid-producing genera that are members of the intestinal microflora has shown that each human harbours their own unique collections of bacterial strains (Box 3)[8]. This technology, combined with the use of polymerase chain reaction methods[9] and oligonucleotide probes[10], now provides the means by which the detailed analysis of the effect of consuming a probiotic product on the compostion of bacterial populations already resident in the intestinal tract can be made.

Choice of strains for use as probiotics

Lactic-acid-producing bacteria are common components of probiotics (Table 1). They are popular choices because of the historical belief that these bacteria are desirable members of the intestinal microflora, arising from the fact that lactic-acid bacteria have long been used in the manufacture of dairy foods and are thus 'generally regarded as safe', and because the consequent large-scale-culture and preservation methods for lactic-acid bacteria in a viable state have already been developed by the dairy industry. The choice of strains to be included in probiotic products has largely been decided on the basis of whether they are amenable to industrial handling and if they will remain viable for a suitable time in the prepared product. While these are legitimate reasons for choosing a strain for industrial use, information as to the ability of the bacterial strain to fulfil the definition of a probiotic (i.e. whether it modifies the intestinal microflora) is, strangely, lacking. Studies demonstrating the recovery of an administered probiotic strain from faeces have been published, but detailed analyses of the microflora following consumption of a commercial product are generally unavailable. Indeed, the detection of probiotic strains may have been somewhat unreliable in studies to date because it has been based on bacterial-colony morphology[11]. However, experimental animal studies conducted in recent years have provided biochemical markers that could be used to show whether an administered probiotic has influenced the intestinal ecosystem. These studies utilized mice that do not harbour lactobacilli as part of their intestinal microflora[12]. Comparisons of the characteristics of *Lactobacillus*-free mice with counterparts that had been intentionally colonized with *Lactobacillus* strains, but in which an otherwise-identical microflora was present, have demonstrated that lactobacilli have major influences on intestinal biochemistry. The biochemical markers identified by these comparisons are bile-salt-hydrolase activity and the concentration of unconjugated bile acids in intestinal contents (increased in the presence of lactobacilli)[13,14], and azoreductase and β-glucuronidase activity (reduced in the presence of lactobacilli)[15,16].

Several *Lactobacillus* species have been included in probiotic products (Table 1). Theoretically, the choice of species should be dictated by the animal species for which the product is intended, because the composition of their microfloras may differ. In practice, comprehensive taxonomic studies based on the molecular phylogeny of lactic-acid components of the intestinal microflora of different animal species have not been made. *Lactobacillus acidophilus*, until recently a catch-all species composed of disparate isolates and used widely to describe the *Lactobacillus* components of probiotics,

has been split into six new groups (*L. acidophilus*, *L. amylovorus*, *L. crispatus*, *L. gallinarum*, *L. gasseri* and *L. johnsonii*)[17]. The exact identity of the lactobacilli included in probiotics is often not known. *Lactobacillus reuteri* is considered by some researchers to be the most prevalent heterofermentative *Lactobacillus* species inhabiting the intestinal tract of humans and other animals[18]. Clearly, extensive research needs to be conducted in this area.

One of the desirable properties sometimes listed for probiotic strains is that they should be resistant to antibiotics[19]. The rationales for this approach are that probiotic products could be used to reconstitute the intestinal microflora of patients suffering from antibiotic-associated colitis or be fed to farm animals administered 'growth-promoting' concentrations of antibiotics in their food. Because a residue of antibiotic could be present in the intestine of the patients or of the farm animals, only antibiotic-resistant probiotic strains would be able to colonize the ecosystem. This approach would surely compound the existing problem of antibiotic resistance in bacteria of medical importance. Although the probiotic bacteria would be unlikely to cause a pathological process, they could serve as a reservoir of antibiotic-resistance determinants that could be transmitted to pathogens. Transmission of antibiotic-resistance determinants on plasmids has been demonstrated to occur from lactobacilli to other Gram-positive bacteria under laboratory conditions and in the intestinal tract[20,21].

Which are best: lactobacilli or bifidobacteria?

Lactobacilli have the longest history of use as probiotics and are still the most common ingredients of those intended for consumption by farm animals, notably pigs and poultry. This choice of probiotic bacteria seems appropriate, because the digestive tract microfloras of these animal species are particularly rich in lactobacilli when the pigs and poultry are maintained under optimal conditions of animal husbandry[22]. *Bifidobacterium* species are now nearly as common as lactobacilli in yogurts, presumably as a result of the realization that the human intestinal tract harbours larger populations of bifidobacteria than lactobacilli. What functions do bifidobacteria perform in the intestinal ecosystem, how do these activities influence the consumer and, therefore, what is the scientific rationale for including them in probiotics? These are, currently, unanswered questions.

The presence of both lactobacilli and bifidobacteria as members of the intestinal tract microflora of the majority of humans logically requires that the ingested probiotic strain have some health-promoting characteristic that the resident lactic-acid bacteria lack. The probiotic strain would need to be able to compete sufficiently with the resident lactobacilli or bifidobacteria for the expression of the beneficial attribute. It is therefore necessary to determine the proportion of the lactobacillar or bifidobacterial population that the probiotic strain achieves in the intestinal tract in order for the product to have scientific validity

Table 1. Examples of microorganisms used in probiotic products

Products for humans	Products for farm animals
Lactobacillus acidophilus	*L. acidophilus*
Lactobacillus casei Shirota strain	*L. casei*
Lactobacillus delbrueckii subspecies *bulgaricus*	*L. delbrueckii* subspecies *bulgaricus*
Lactobacillus johnsonii	*L. plantarum*
Lactobacillus reuteri	*L. reuteri*
Lactobacillus rhamnosus	*Bifidobacterium bifidum*
Bifidobacterium adolescentis	*Bacillus subtilis*
Bifidobacterium bifidum	*Streptococcus thermophilus*
Bifidobacterium breve	*Pediococcus pentosaceus*
Bifidobacterium longum	*Enterococcus faecium*
Bifidobacterium infantis	*Saccharomyces cerevisiae*
Streptococcus thermophilus	*Aspergillus oryzae*
Saccharomyces boulardii	*Torulopsis* spp.

Probiotics and the immune system

While the purported benefits of the consumption of probiotic products are several, and often astounding[23], the relationship between microorganisms inhabiting the intestinal tract and the immune system of their host can be investigated scientifically. Interest in the ability of lactic-acid-producing bacteria to stimulate the defence mechanisms of the body originated in the use of extracts of fermented mistletoe to treat cancer patients in the 1920s. The extracts were demonstrated to stimulate the immune system of experimental animals; the stimulating components were the cells of lactobacilli[24]. This immunological effect, which principally involves the activation of macrophages, is due to constituents of the bacterial cell wall[25]. Lactobacilli are Gram-positive bacteria and thus their cell wall is composed mostly of peptidoglycan. Degradation products of peptidoglycan include muramyl peptides, which can be detected in the systemic tissues[26] and have pharmacological activity affecting sleep patterns, body temperature and appetite[27–29]. Additionally, muramyl peptides are strong adjuvants, as can be seen from the fact that they enhance immunological reactivity[30].

Lactobacilli, and other members of the intestinal microflora present in the digestive tract contents, are separated from the tissues and organs of the host by the intestinal epithelium. The epithelium is composed of a layer, only one cell thick, of column-shaped cells referred to as enterocytes. How do *Lactobacillus* cell wall substances and other constituents gain access to the immune system of the host? Perhaps the most probable explanation is the passage of particulate or soluble *Lactobacillus* substances into the accumulations of immunological tissue, the Peyer's patches that are present at intervals along the intestinal wall[31]. The patches are separated from the intestinal lumen by M cells that appear to have an antigen-sampling role. Whole bacterial cells may also pass (translocate) from the intestinal lumen into the blood or lymphatic systems via the Peyer's patches or temporary breaks in the epithelial barrier. Bacterial translocation is rare in

healthy, adult animals but may be more common in young animals[32]. The lactobacilli and other microorganisms may be able to stimulate the immune system without leaving their lumenal habitat since enterocytes, it is now recognized, are immunocompetent cells and produce chemical messengers (cytokines) involved in the regulation of the immune system. When exposed to certain bacterial cells or their products, enterocytes have been shown to increase the expression of genes encoding interleukin 8, tumour necrosis factor α, monocyte chemotactic protein 1 and granulocyte–macrophage colony-stimulating factor[33]. These substances have well-established roles in the attraction and activation of polymorphonuclear leukocytes and macrophages. Investigation of the effect of *Lactobacillus* strains on the expression of cytokines by enterocytes should therefore be an area of high priority for probiotic research.

Conclusion and prospects

The derivation of efficaceous probiotics requires substantial research and development, especially at the level of fundamental science. An investment by biotechnological companies in obtaining knowledge of the microbial ecology of the intestinal tract will pay dividends for them, because the results of this research will provide a basis for firm claims of efficacy to be made in the marketing of probiotic products. The incorporation of intestinal strains of lactic-acid bacteria into yogurts provides the opportunity to market a 'functional food': one that combines nutrition with another beneficial consequence for the consumer.

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